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Restricted antigenic variability of the epitope recognized by the neutralizing gp41 antibody 2F5

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Objective: To investigate whether variations of the conserved gp41 amino-acid sequence ELDKWA affect its binding or neutralization by monoclonal antibody (MAb) 2F5.

Design and methods: Neutralization assays were performed with primary isolates from different HIV-1 subtypes and the sequences corresponding to the 2F5 epitope region were analysed. Studies of MAb 2F5 peptide reactivity were performed by spot analysis, using peptides immobilized on cellulose. The frequency of emergence of neutralization-resistant virus variants was determined by immune selection experiments in the presence of MAb 2F5.

Results: Primary isolates from clades A, B and E were neutralized by MAb 2F5. Neutralization sensitivity correlated with the presence of the LDKW motif. A K-to-N change in the core sequence was identified in a neutralization-resistant patient isolate. Neutralization resistant virus variants that were selected in the presence of MAb 2F5 were found to contain D-to-N, D-to-E, or K-to-N changes within the LDKW sequence. Neither in natural isolates nor in variants obtained under immune selection conditions in the laboratory were changes in the L and W positions observed. Studies of MAb 2F5 binding to variations of the ELDKWA peptide confirmed that the changes at the first and last positions did not significantly reduce binding capacity, whereas amino-acid changes from D to N, D to E, and K to N almost completely abrogated binding of MAb 2F5.

Conclusion: Sequence analysis of a variety of primary isolates suggests that the major determinant of MAb 2F5 binding corresponds to the amino-acid sequence LDKW. Naturally occurring and *in vitro* selected neutralization-resistant viruses contained changes in the D and K positions of the ELDKWA motif.

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Introduction

The creation of an effective, humoral immunity-based vaccine against HIV-1 would be assisted by the identification of monoclonal antibodies (MAb) with a potent virus-neutralizing capacity and by the characterization of the viral determinants that are the targets of these antibodies. Many MAb recognizing diverse regions of the envelope glycoproteins gp120 and gp41 of HIV-1 have been described to have the ability to neutralize

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transformed T-cell-line-adapted virus variants. However, cell-line-adapted viruses are more sensitive to neutralization by soluble CD4 and polyclonal antibodies as well as MAb than primary HIV-1 isolates [1,2]. Because primary viruses, grown solely on peripheral blood mononuclear cells (PBMC), more closely reflect the virus strains found *in vivo*, a major criterion for a MAb to be included as a reagent for immunoprophylactic or immunotherapeutic strategies is therefore the breadth and potency of its activity against this kind of viruses. The classification of HIV-1 into subtypes (clades) A to H and O [3] demonstrates the broad heterogeneity of this lentivirus group. The highest degree of heterogeneity has been described for the *env* gene [4,5], most of the amino-acid heterogeneity being found in the five variable regions of gp120 (V1–V5). Nevertheless, highly conserved regions on gp120/41 can be identified as targets of neutralizing antibodies that mediate cross-clade activity [6]. One of these highly conserved epitopes is the amino-acid sequence ELDKWA on the ectodomain of transmembrane glycoprotein gp41 [7]. This epitope is recognized by the human MAb 2F5 which has been shown to neutralize a broad variety of T-cell-line-adapted and primary HIV-1 variants of different clades [6,8,9].

In this study we characterize certain immunochemical properties of MAb 2F5 and its peptide epitope. Specifically, we analysed the binding pattern of MAb 2F5 to a series of variants of the original ELDKWA peptide. Moreover, we investigated the antigenic variability of this epitope region in immune selection experiments. Neutralization escape variants that were generated in the presence of MAb 2F5 were analysed and neutralization resistance was found to correlate with the occurrence of amino-acid substitutions in the ELDKWA sequence. Naturally occurring and *in vitro*-selected escape variants revealed changes in only two positions of the ELDKWA sequence. The predominant changes were D to N and K to N.

Materials and methods

Antibodies, viruses and cells

The production of human MAb 2F5 and 3D6 has been described previously [10,11]. MAb 3D6 recognizes a linear epitope in the immunodominant region of gp41 (amino acids 597–609 according to gp160 sequence of HTLV-IIIB clone BH10) and binds to a broad variety of HIV-1 isolates [11,12]. The cell line H9/HTLVIIIB was obtained from the American Type Culture Collection. The cell lines AA-2 [13] and H9 [14] were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, US National Institutes of Health). The molecular HIV-1 clone cl82 was kindly provided by E.M. Fenyö (Karolinska Institute, Stockholm, Sweden) [15]. Primary isolates WYG, WRF, WHM, WRB and WSC were isolated from Austrian patients with varying disease status, as described previously [8]. Virus isolates designated by a code in the format exemplified by 92BR021 were obtained by the World Health Organization Network for HIV Isolation and Characterization [16,17]. Cultivation of non-infected and infected cell lines, preparation of mitogen-stimulated PBMC and production of virus stocks were performed as previously described [8].

Syncytium inhibition assays

Syncytium inhibition was assessed using AA-2 cells as indicator cell line with syncytium formation determined by light microscopy as described previously [8]. Prior to the experiments, virus stocks were titrated on AA-2 cells and the 50% tissue culture infective dose ($TCID_{50}$) was calculated by the method of Reed and Muench [18]. Serial dilutions of MAb were pre-incubated with virus (10^2–10^3 $TCID_{50}$/ml) for 1 h at 37°C before AA-2 cells were added. After 5 days incubation, syncytium formation was assessed. The presence of one syncytium per culture was considered as an indication of HIV-1 infection. The 50% effective concentration (EC_{50}) was then calculated by the method of Reed and Muench [18]. EC_{50} is defined as the antibody concentration resulting in 100% inhibition of syncytium formation in 50% of the wells. A titration of the virus inoculum was included in each experiment.

Neutralization assays

Neutralization assays were performed with phytohaemagglutinin (PHA)-stimulated PBMC from healthy HIV-seronegative blood donors on HIV susceptible cells with p24 antigen production as virus replication marker, as described previously [8]. Antibodies were adjusted to 100 µg/ml and 50 µl twofold serial dilutions were incubated with 50 µl virus inoculum (10^2–10^3 $TCID_{50}$/ml) for 1 h at 37°C; 100 µl PHA-stimulated PBMC at a density of 4×10^6/ml were added to the MAb/virus mixture and cultivated for 7 days. All assays were performed with four replicates per dilution step. The cultures were collected and treated with Nonidet P-40 detergent (2% final concentration), and replicate wells of each MAb concentration were pooled before determination of p24 production [6,8] so that interwell variation was minimized. The ratios of p24 antigen production in MAb-containing cultures to p24 antigen production in control cultures were estimated, and the MAb concentrations (µg/ml) causing 50, 90 and 99% inhibition (IC_{50}, IC_{90} and IC_{99}, respectively) were determined by linear regression analysis. Tabulated values are mean values plus SD from two to four independent experiments. All neutralization assays included a titration of the virus inoculum to confirm the actually used infectious titre.

Immune selection experiments

HIV-1 isolate IIIB and the molecular clone cl82 were diluted to 10^2–10^3 $TCID_{50}$/ml and pre-incubated with different concentrations of MAb 2F5 for 1 h at 37°C

before AA-2 cells were added. Cells were also infected and cultivated in the absence of antibody. Twice-weekly cultures were fed with medium containing the appropriate amount of antibody, and production of p24 antigen in the supernatant was monitored. Supernatants from cultures where virus had emerged were harvested and cultivated with AA-2 cells in the absence of MAb to produce stocks of these virus variants. Cell-free supernatant from these virus production cultures was harvested after 1 week and stored in aliquots at -80°C. Supernatant from the control cultures was also harvested to produce virus stocks, to identify whether mutations could occur in the absence of antibody pressure. Finally, the isolated virus variants were titrated and characterized for their sensitivity to neutralization by MAb 2F5 in syncytium inhibition assays.

Immunocapture and sequence analysis

An immunocapture reverse transcription polymerase chain reaction (RT–PCR) method modified from Brandt *et al.* [19] was performed. Polypropylene microcentrifuge tubes (0.5 ml, MultiTechnology, Inc., Salt Lake City, Utah, USA) were coated with 10 µg/ml anti-gp41 antibody 3D6 in coating buffer (0.1 M sodium carbonate buffer, pH 9.6). After 2 h at room temperature unbound antibody was removed by two washes with phosphate-buffered saline (PBS). Virus particles from cell-free supernatant were pelleted in 3 ml polyallomer centrifuge tubes at 70 000 r.p.m. for 65 min at 4°C (Beckman ultra centrifuge TL100 with rotor TLA100.3; Palo Alto, California, USA). The pellet was resuspended in 100 µl PBS and incubated for 2 h at 4°C in the 3D6-coated tubes. Bound virus particles were washed twice with PBS and then lysed with 0.1% Triton X-100. Reverse transcription was performed directly in the immunocapture tubes with the antisense primer corresponding to an *Eco*RI restriction site and nucleotide positions 2034 to 2013 of BH10 gp160 as described elsewhere [7]. PCR was performed with the antisense primer and the sense primer corresponding to a *Bam*HI restriction site and nucleotide positions 1948 to 1918. The resulting PCR products were digested with *Bam*HI and *Eco*RI, cloned into the plasmid p-Gex-2T (Pharmacia, Uppsala, Sweden), and sequenced by the dideoxy-chain termination method.

Peptide spot analysis

To identify tolerated and sensitive amino-acid substitutions in the epitope of MAb 2F5, we performed binding studies using the peptide ELDKWA and hexamer peptides derived from this sequence but containing single amino-acid substitutions. These were made using a spot synthesis protocol [20]. In this procedure, peptides were synthesized *in situ*, carboxy-terminally bound to cellulose on a 6×17 cm Whatman 540 paper-sheet (Maidstone, England, UK). Each amino acid of the six amino-acid peptide ELDKWA was substituted by all amino acids. Each spot represents about 50 nmol of peptide. After synthesis, cellulose-bound peptides were washed three times with T-PBS (PBS with 0.1% Tween-20) and incubated overnight with Superblock blocking buffer (Pierce, Rockford, Illinois, USA). Finally, the sheet was washed twice with T-PBS and incubated with 2 µg/ml MAb 2F5 in T-PBS containing 1% skim milk powder for 2 h. Bound antibody was detected with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Sigma, St Louis, Missouri, USA). Staining solution (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in 0.15 M veronal acetate buffer, pH 9.6) was added and the reaction was stopped after 5 min by rinsing the paper-sheet with PBS containing 20 mM EDTA.

Results

Neutralization of primary viruses

To examine the neutralization capacity of MAb 2F5 against primary isolates of different clades, we performed neutralization assays using PHA-stimulated PBMC. Altogether we tested 13 isolates, three from clade A, three from clade B, two from clade E, and five primary isolates from Austrian patients that have yet not been subtyped. Neutralization titres for these isolates are listed in Table 1. MAb 2F5 reduced the infectivity of 12 out of the 13 tested isolates by 90% at concentrations < 50 µg/ml. Seven of these isolates were neutralized by 99%. Only one of the tested isolates (WSC) was resistant to neutralization by MAb 2F5.

Immune selection

We performed immune selection experiments with the molecular HIV-1 clone cl82 at MAb 2F5 concentrations of 25, 12.5, 6.3, 3.2, 1.6, 0.8 and 0.4 µg/ml. Several neutralization-resistant virus variants emerged within a period of 3–12 weeks after infection in cultures containing 1.6, 0.8 and 0.4 µg/ml MAb (Table 2). Virus production in control cultures was observed within 1 week. No virus production could be detected when 25, 12.5, 6.3 or 3.2 µg/ml MAb 2F5 were added to the cultures. When we tested the immune-selected viruses for their sensitivity to neutralization by MAb 2F5 we found that the variants that escaped the antibody pressure could not be neutralized by MAb 2F5 at the highest concentration tested (50 µg/ml), whereas the virus isolated from the control culture was still neutralized by 2F5 to the same extent as the original wild-type cl82 (EC_{50} of 1.6 µg/ml). In immune selection experiments with isolate IIIB, no virus emerged in the presence of 50 and 10 µg/ml MAb 2F5 (data not shown), whereas virus production was detected after 3 weeks in cultures that contained 1.6 µg/ml MAb 2F5. This also corresponds approximately to the EC_{50} concentration for isolate IIIB. Virus production was observed within 1 week in control cultures. (The selection conditions and sequence analysis of this neutralization-resistant virus variant were previously described by Muster *et al.* [7]; a summary is

Table 1. Neutralization titers of monoclonal antibody 2F5 against primary HIV-1 isolates.

Isolate	Clade	Mean ± SD* (μg/ml)		
		IC_{99}	IC_{90}	IC_{50}
92RW009	A	28.8 ± 14.0	7.3 ± 1.2	0.3 ± 0.2
92RW021	A	40.5 ± 8.6	4.8 ± 3.1	0.7 ± 0.8
92UG037	A	> 50.0	16.7 ± 7.0	0.7 ± 0.4
92TH014	B	40.5 ± 12.7	21.4 ± 6.3	2.6 ± 1.2
92BR021	B	28.3 ± 6.5	10.1 ± 1.5	2.7 ± 0.4
92BR030	B	> 50.0	32.7 ± 8.1	5.0 ± 0.8
92TH021	E	> 50.0	16.6 ± 5.2	1.1 ± 0.6
92TH024	E	8.7 ± 2.1	0.3 ± 0.3	0.01 ± 0.05
WYG	ND	21.5 ± 5.2	5.0 ± 2.2	0.8 ± 0.6
WRF	ND	> 50.0	36.0 ± 10.2	4.7 ± 1.9
WHM	ND	48.4 ± 7.4	26.5 ± 6.0	2.1 ± 2.3
WRB	ND	> 50.0	40.9 ± 10.6	5.4 ± 4.5
WSC	ND	> 50.0	> 50.0	> 50.0

ND, not determined. IC_x, x% inhibitory dose. *Means ± SD derived from two to four independent experiments.

included in Table 2.) Virus variants selected in the presence of MAb 2F5 were neutralization-resistant to MAb 2F5 but retained their neutralization sensitivity to the gp120 MAb 1B1 (directed at the CD4-binding site) [11] and 2G12 (recognizing a conformational, glycan-dependent epitope) [21] (data not shown).

Table 2. Neutralization-resistant virus variants to monoclonal antibody (MAb) 2F5.

	Selection conditions			Epitope sequence					
Virus variant	HIV-1 strain	2F5 (μg/ml)	No. clones	E	L	D	K	W	A
C/1	cl82	1.6	2/2	–	–	N	–	–	–
C/2	cl82	0.8	4/5	–	–	N	–	–	–
			1/5	–	–	–	N	–	–
C/3	cl82	0.4	2/5	–	–	–	N	–	–
			2/5	–	–	N	–	–	–
			1/5	–	–	–	–	–	–
B/1	IIIB	1.6	2/4	–	–	N	–	–	–
			1/4	–	–	E	–	–	–
			1/4	–	–	–	–	–	–

Immune selection experiments were performed with the molecular clone cl82 (this study) and HIV-1 isolate IIIB (see Muster *et al.* [7]) and with MAb 2F5. Only the selection conditions which led to virus production are listed. Sequence analysis of the 2F5 epitope region was performed from two to five clones of each escape virus variant.

Sequence analyses

Sequence analysis of the 2F5 epitope region was performed for the neutralization-resistant virus variants. The observed nucleotide changes resulted in the amino-acid changes shown in Table 2. Each of the escape variants showed changes in the D or K positions of the epitope sequence. The repeatedly observed amino-acid changes from D to N and from K to N as well as the D-to-E change, resulted from single point mutations. Most of the virus variants also contained clones that had the unchanged epitope sequence suggesting that the neutralization-resistant virus strain contained a mixture of phenotypes. The epitope sequences of the primary virus isolates used in neutralization assays were also analysed (Table 3). Neutralization-sensitive viruses contained the ELDKWA sequence, with a few amino-acid substitutions tolerated in the first and last positions (Table 3). Substitution of E to A/G/V and of A to E/S within the ELDKWA sequence did not result in alteration of the sensitivity of MAb 2F5. However, isolate WSC, that could not be neutralized, had the sequence ALDNWA within the 2F5 epitope; the K-to-N change probably accounts for its 2F5 neutralization resistance.

Table 3. Sequences of the 2F5 epitope region in tested primary viruses.

Isolate	Clade	Neutralized	Epitope sequence					
			E	L	D	K	W	A
92RW009	A	+	–	–	–	–	–	–
92RW021	A	+	–	–	–	–	–	–
92UG037	A	+	–	–	–	–	–	–
92TH014	B	+	–	–	–	–	–	–
92BR021	B	+	–	–	–	–	–	–
92BR030	B	+	G	–	–	–	–	–
92TH021	E	+	–	–	–	–	–	–
92TH024	E	+	–	–	–	–	–	–
WYG	ND	+	–	–	–	–	–	E
WRF	ND	+	V	–	–	–	–	S
WHM	ND	+	A	–	–	–	–	–
WRB	ND	+	–	–	–	–	–	–
WSC	ND	–	A	–	–	N	–	–

ND, not determined.

Sequence comparison

Comparison of the gp41 amino-acid sequences of 96 virus isolates described in the Los Alamos database [3] indicated a high degree of conservation of the LDKW motif (82%). The L and W positions were found in 100% of the isolates. Only one isolate showed a D-to-N substitution. The K position revealed a moderate degree of variability with alterations to Q, T, E, N, and S.

Peptide spot analysis

To identify amino-acid changes in the epitope of MAb 2F5 that affect recognition by the antibody, binding studies with peptides which contain single amino-acid substitutions in positions 1–6 of the ELDKWA epitope sequence were performed. Amino acids in each position were systematically substituted by all proteinogenic amino acids. The binding capacity of MAb 2F5 to the mutant peptides is shown in Fig. 1. Substitutions of E in the first position of the epitope sequence did not significantly alter the binding properties of MAb 2F5. Simi-

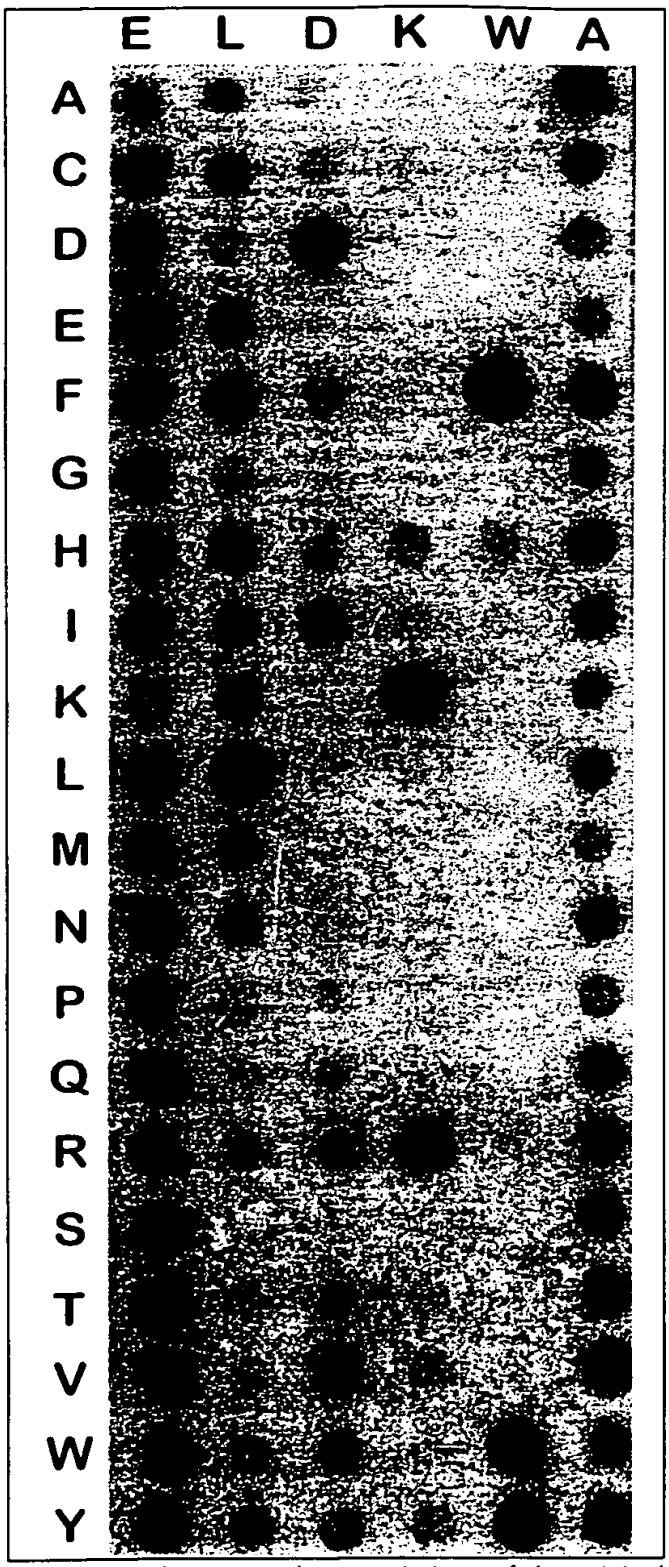

Fig. 1. Peptide spot analysis. Variations of the original ELDKWA peptide sequence with single amino-acid substitutions in position 1 to 6 were synthesized on cellulose. Columns (E, L, D, K, W, A) indicate which amino acid had been changed; rows (A–Y) indicate the amino acid used in exchange for the original one. Binding of MAb 2F5 to the immobilized hexamer peptides was studied.

larly, substitutions of A in the last position showed only minor effects on antibody binding. Some substitutions (to F, H or K) are tolerated for the L but binding was almost completely abrogated when the amino acids D, K or W were changed. This binding pattern is consistent with the observations described by Muster *et al.* [7] and Conley *et al.* [9].

Discussion

In the present study the breadth of reactivity of MAb 2F5 was attested by the antibody's significant neutralization potency against African, Asian, American and European strains from clades A, B and E. Most of the investigated viruses were neutralized by 90% at concentrations that could be achieved *in vivo* by exogenous MAb addition. Clonotype concentrations of antibodies in patient sera have been shown to range between 10 and 50 μg/ml [22,23]. Sequence analysis of the tested viruses revealed that the presence of the ELDKWA epitope sequence was correlated with neutralization sensitivity. Amino-acid variations in the first and last positions did not significantly influence the virus-inhibiting properties of MAb 2F5, whereas substitutions of the D and K positions were found only in neutralization-resistant isolates. Some isolates, for instance 92TH024 and WRB, differ significantly in their sensitivity to neutralization by MAb 2F5 despite sharing the identical epitope sequence. Sequence variation outside of the linear epitope might alter the overall three-dimensional conformation of the protein and influence the accessibility of the ELDKWA region which results in the observed differences in neutralization sensitivity. It was shown recently that 2F5 reactivity to native oligomeric gp160 is decreased after soluble CD4 (sCD4) binding to gp120 [24]. This might be an indication that receptor binding induces a conformational modification of the epitope or leads to epitope masking after receptor-induced interaction of gp41 with other molecules on the infected cell or virion membranes. Another study could demonstrate that binding of 2F5 to virions reduces the accessibility of the CD4 binding site [25]. Binding of sCD4 to monomeric gp160, however, is not affected by prior incubation with 2F5 (data not shown). In the present stage it cannot be excluded that MAb 2F5 binds in addition to regions present on the oligomeric form of the envelope protein. The ELDKWA peptidic structure, however, represents the essential part of the 2F5 epitope as was already demonstrated elsewhere [7,9]. The relative resistance of some isolates to neutralization by MAb 2F5 might also be due to differences in virus growth characteristics caused by changes in other parts of the genome. The observed differences in neutralization titres are not a discrepancy but expected when comparing highly divergent HIV-1 isolates.

The naturally occurring isolates found to be resistant to neutralization by MAb 2F5 had amino-acid changes in the core epitope sequence from K to N (WSC, Table 3; and SG364 [6]) and from K to E (MVP5180 [6]). Similar changes have been observed in the immune-selected virus variants that have been obtained from laboratory adapted virus on AA-2 cells (Table 2).

Furthermore, the presence of the LDKW sequence was found to be crucial for recognition of peptides when the antibody was tested in binding experiments. The LDKW motif was found in 82% of the virus sequences described in the Los Alamos database [3], whereas the amino acids L and W were found to be 100% conserved. Moreover, none of the escape mutants generated in immune selection experiments had introduced changes of either of these two amino acids. Changes at these positions may be incompatible with virus replication. Emergence of neutralization-resistant HIV-1 variants in the presence of neutralizing antibodies is frequently observed *in vivo* [26,27] and *in vitro* [28–31]. In immune selection experiments with MAb 2F5 and the laboratory adapted HIV-1 isolates IIIB and cl82, virus escape was observed only at subneutralizing concentrations of MAb 2F5. Higher concentrations of MAb 2F5 are expected to be needed to prevent emergence of escape mutants with primary isolates as in general primary viruses are more resistant to neutralization.

The amino-acid changes obtained *in vitro* as well as the substitutions observed in the databases from K to Q/T/E/N result from point mutations, whereas several C-clade viruses contained a K-to-S mutation. The limited occurrence of mutants in this gp41 epitope *in vitro* and *in vivo* might be an indication, that this region plays an important role in viral replication cycle and only few changes are tolerated to retain full functionality. With respect to the creation of a broadly reactive vaccine against HIV-1 infection it would seem useful to present the ELDKWA sequence, as well as the closely related naturally occurring variants of this peptide sequence, to the immune system to induce a 2F5-like immune response. Recently, we demonstrated that the ELDKWAS epitope sequence, when presented in antigenic site B of influenza virus hemagglutinin, is able to induce neutralizing antibodies against different HIV-1 laboratory strains [32]. However, we could not induce antisera that were effective against primary isolates, which indicates that the epitope sequence is not presented in its optimal conformation in this construct. Therefore, our current strategies are to express the 2F5 epitope in a variety of different conformations and to analyse their ability to induce antisera that effectively neutralize primary HIV-1 isolates.

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